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COMPLETE SPECIFICATION

Aqueous Lysozyme Solution and method of making the same

We, EISAI CO. LTD., a Japanese Corporation, of 16, 6-ban, 4-chome, Koishikawa, Bunkyo-ku, Tokyo, Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to an aqueous lysozyme solution and to a method of making the same.

Lysozyme is a cationic protein discovered as a bacteriolytic enzyme of egg white. It hydrolyzes the $\beta(1\text{—}4)$ linkage of mucopolysaccharide and mucopeptide. It is also called muramidase.

This enzyme is widely distributed not only in egg white but also in body fluids, tissues and organs of animals and human beings. When lysozyme is orally or parenterally administered, it shows a wide range of pharmacological properties such as anti-oedematic and antiphlogistic actions, an antiviral action, a hemostatic action and normalising action on the enteric flora. Therefore, it is widely used for therapeutic purposes.

However, it has been difficult to make an aqueous preparation of lysozyme or its salt because the enzyme in aqueous solution undergoes surface denaturation on filtration or shaking and the denatured lysozyme is precipitated with resulting inactivation.

It is an object of the present invention to provide a method of preventing the formation of a flocculent precipitate of denatured lysozyme in an aqueous solution of lysozyme and/or a salt thereof, which method avoids the above-mentioned disadvantages. It is a further object of the present invention to provide a composition comprising an aqueous solution of lysozyme and/or salts thereof.

According to one aspect of the present invention there is provided a method of preventing the formation of a flocculent precipitate of denatured lysozyme in an aqueous solution of lysozyme and/or a salt thereof, which method comprises adding to said aqueous solution a nonionic surface active agent, which is dispersible or soluble in water in a quantity sufficient to make its final concentration in said solution more than 0.0005% weight/volume.

According to another aspect of the present invention there is provided a composition comprising an aqueous solution of lysozyme and/or salts thereof, said solution containing more than 0.0005% weight/volume of a nonionic surface active agent which is dispersible or soluble in water.

Because changing the physical condition of the molecular surface of lysozyme in aqueous solution might prevent denaturing, the invention is based on a study of means for preventing such denaturing and the accompanying precipitation. In this study various surface active agents were added at various concentrations to aqueous solutions of lysozyme and the degree of the production of flocculent precipitate as a result of shaking the solution with an Iwaki shaker was observed. It was discovered that precipitation could be prevented by adding a very small amount of a nonionic surface active agent.

Testing solutions were prepared by adding various surface active agents at various concentrations to an aqueous solution of lysozyme of the composition shown below. 10 ml. of each test solution was placed in an ampoule of a capacity of 20 ml., the ampoules were sealed by fusing, each ampoule was mechanically shaken (360 times per minute with 5 cm amplitude for 24 hours) and the formation of flocculent precipitate in each ampoule was observed. The result is shown in Table 1.

Basic formula:	By weight
Lysozyme chloride	2.50%
Sorbitol	5.00
Methyl paraben	0.13
Propyl paraben	0.02
Distilled water to make the total amount	100.00

[*Price 4s. 6d.*]

TABLE I

Experimental No.	Added surface active agent	HLB	Concentration of surface active agent (in % weight/volume)	Formation of precipitate after shaking
1	Non	—	—	++
2	Polyoxyethylene—(10)—stearate	10.8	0.1	+
3	„ —(25)— „	15.2	0.1	—
4	„ —(40)— „	17.4	0.1	—
5	„ —(45)— „	17.9	0.1	—
6	„ —(55)— „	19.1	0.1	—
7	Hydrogenated castor oil poloxyethylene —(20)—ether	9.2	0.1	—
8	„ —(40)— „	12.3	0.1	—
9	Hydrogenated castor oil polyoxyethylene—(60)—ether	14.1	1.0	—
10	„ „ „ „	14.1	0.5	—
11	„ „ „ „	14.1	0.25	—
12	„ „ „ „	14.1	0.1	—
13	„ „ „ „	14.1	0.05	—
14	„ „ „ „	14.1	0.005	—
15	„ „ „ „	14.1	0.0005	++
16	„ „ —(80)— „		0.1	—
17	„ „ —(100)— „		0.1	—
18	Polyoxyethylene sorbitan monooleate	15.0	0.1	—
19	Polyoxyethylene sorbitan trioleate	11.0	0.1	+
20	Sodium lauryl sulphate		0.1	+++
21	Benzethonium chloride		0.1	*
22	Polyoxyethylene nonyl phenol formaldehyde condensate	13.2	0.1	+
23	Polyglycerol lauric acid ester	16.0	0.1	—

+++ A large amount of a precipitate was formed immediately on addition of the sodium lauryl sulphate to the lysozyme solution.
++ A large amount of precipitate was formed after shaking.
+ As the surface active agent was not completely dissolved and the solution was cloudy, the formation of precipitate of lysozyme was difficult to observe.
— The precipitate was not observed.
* The precipitate was not observed, but the solution became turbid.

In Table 1, HLB is the Hydrophile-Lipophile-Balance of surface active agent, the explanation of which is found in the following literatures: W. C. Griffin: J. Soc. Cosm. Chem., Vol. 1, p. 311 (1949), ibid. Vol. 5, p. 1., (1954).

An anionic surface active agent, sodium lauryl sulfate, forms an insoluble complex with lysozyme. A cationic surface active agent, benzethonium chloride, is seen to be effective but it tends to make the solution turbid and, therefore, it has little pharmaceutical use.

In carrying out the present invention, all kinds of nonionic surface active agents, which are dispersible or soluble in water, and which have HLB values of about 10 or more, are effective above the aforesaid concentration.

As nonionic surface active agents the following may be used, alkyl or alkyl aryl polyoxyethylene ethers such as polyoxyethylene - (25) - lauryl ether and polyoxyethylene - (20) - nonyl phenol ether, polyoxyethylene fatty acid esters such as polyoxyethylene - (25) - stearate, polyoxyethylene sorbitan fatty acid esters such as polyoxyethylene sorbitan monooleate, polyoxyethylene alkyl phenol formaldehyde condensates such as polyoxyethylene nonyl phenol formaldehyde condensate, polyoxyethylene alkyl amines such as N,N - dipolyoxyethylene-(20) - lauryl amine, polyoxyethylene alkyl amides such as N,N - dipolyoxyethylene-(20) - lauryl amide, polyoxyethylene - polyoxypropylene block polymers, hydrogenated castor oil polyoxyethylene ethers such as hydrogenated castor oil polyoxyethylene - (60) - ether, and polyglycerol fatty acid esters such as polyglycerol lauric acid ester (HLB 16). Under the above mentioned experimental condition, nonionic surface active agent below the concentration of 0.0005% by wt/volume was not effective for preventing the formation of precipitate of denatured lysozyme from the aqueous solution, but below the concentration of 0.0005% wt/volume an effect is evident. Accordingly, the concentration of a non-ionic surface active agent should be more than 0.0005% (W/V), and so, nonionic surface active agents at the final concentration of more than 0.005% and less than 1.0% (W/V) are suitable for practical use.

This invention may be used to make the aqueous solution of lysozyme and/or its salts preferably of a concentration below ten percent (W/V). The influences of nonionic surface active agents on the enzymic activity of lysozyme has also been investigated. That is to say, when various nonionic surface active agents were added in several concentrations between 0.001 and 10.0% by wt/volume to aqueous solutions containing 2.5% by wt/volume lysozyme, the lysozyme was not inactivated in each case. The enzymatic activity of lysozyme was determined by measuring its bacteriolytic action on *Micrococcus lysodeikticus* ATCC 4698 by turbidimetry.

EXAMPLE 1:

	Concentration by wt/volume
Lysozyme chloride	2.5%
Sorbitol	5.0
Hydrogenated castor oil polyoxyethylene-(60)-ether	0.005
Methyl paraben	0.13
Propyl paraben	0.02
Distilled water to make the total amount	100.00

An aqueous solution of the above composition was filtered through bacteria-retaining filters under aseptic condition. A sterile ampoule of a 2 ml. capacity was filled with 2 ml. of said solution and was sealed by fusing. The solution is suitable for intramuscular injection.

EXAMPLE 2:

	Concentration by wt/volume
Lysozyme chloride	2.5%
Sorbitol	5.0
Polyexyethylene sorbitan-monooleate	0.1
Methyl paraben	0.13
Propyl paraben	0.02
Distilled water to make the	
Distilled water to make the total amount	100.00

An aqueous solution of the above recipe was filtered through bacteria-retaining filters under aseptic condition. A sterile ampoule of a 2 ml. capacity was filled with 2 ml. of said solution and was sealed by fusing. The solution is suitable for intramuscular injection.

EXAMPLE 3:

	Concentration by wt/volume
Lysozyme	2.5%
Sorbitol	5.0
Polyexyethylene-(40)-stearate	0.1
Methyl paraben	0.13
Propyl paraben	0.02
Distilled water to make the total amount	100.00

An aqueous solution of the above composition was filtered through bacteria-retaining filters under aseptic condition. A sterile ampoule of a 2 ml. capacity was filled with 2 ml. of said solution and was sealed by fusing. The solution is suitable for intramuscular injection.

EXAMPLE 4:

	Concentration by wt/volume
Lysozyme	1.5%
Glucose	5.0
Hydrogenated castor oil polyoxyethylene-(40)-ether	0.25
Methyl paraben	0.13
Propyl paraben	0.12
Distilled water to make the total amount	100.00

A preparation of the above composition was filtered through bacteria-retaining filters under aseptic condition. A sterile ampoule of a 5ml. capacity was filled with 5 ml. of said preparation and was sealed by fusing.

WHAT WE CLAIM IS:—

1. A method of preventing the formation of a flocculent precipitate of denatured lysozyme in an aqueous solution of lysozyme and/or a salt thereof, which method comprises adding to said aqueous solution a nonionic surface active agent, which is dispersible or soluble in water in a quantity sufficient to make its final concentration in said solution more than 0.0005% weight/volume.

2. A composition comprising an aqueous solution of lysozyme and/or salts thereof, said solution containing more than 0.0005% weight/volume of a nonionic surface active agent which is dispersible or soluble in water.

3. A method as claimed in claim 1 or a composition as claimed in claim 2 in which the nonionic surface active agent is an alkyl or alkyl aryl polyoxyethylene ether, a polyoxyethylene fatty acid ester, a polyoxyethylene sorbitan fatty acid ester, a polyoxyethylene alkyl phenol formaldehyde condensate, a polyoxyethylene alkyl amine or amide, a polyoxyethylene-polyoxypropylene block polymer or a hydrogenated castor oil polyoxyethylene ether.

4. A method as claimed in claim 1 or a composition as claimed in claim 2 in which the nonionic surface active agent is a fatty acid ester of polyglycerol.

5. A method of preventing the formation of a flocculent precipitate of denatured lysozyme in an aqueous solution of lysozyme and/or a salt thereof, said method being as claimed in claim 1 and substantially as herein described with reference to the Examples.

6. A composition as claimed in claim 2 when prepared by the method claimed in claim 1 or any one of claims 3 to 5.

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